Serglycin Proteoglycan Promotes Apoptotic *versus* Necrotic Cell Death in Mast Cells*

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Background: Serglycin is a secretory granule proteoglycan with a role in intracellular storage.
Results: In response to cell death-inducing agents, wild type mast cells die by apoptosis whereas serglycin$^{-/-}$ cells undergo necrosis.
Conclusion: Serglycin promotes apoptotic *versus* necrotic cell death in mast cells.
Significance: The present study implicates a pathway involving the secretory granule compartment in regulation of cell death.

The mechanisms that govern whether a cell dies by apoptosis or necrosis are not fully understood. Here we show that serglycin, a secretory granule proteoglycan of hematopoietic cells, can have a major impact on this decision. Wild type and serglycin$^{-/-}$ mast cells were equally sensitive to a range of cell death-inducing regimens. However, whereas wild type mast cells underwent apoptotic cell death, serglycin$^{-/-}$ cells died predominantly by necrosis. Investigations of the underlying mechanism revealed that cell death was accompanied by leakage of secretory granule compounds into the cytosol and that the necrotic phenotype of serglycin$^{-/-}$ mast cells was linked to defective degradation of poly(ADP-ribose) polymerase-1. Cells lacking mouse mast cell protease 6, a major serglycin-associated protease, exhibited similar defects in apoptosis as observed in serglycin$^{-/-}$ cells, indicating that the pro-apoptotic function of serglycin is due to downstream effects of proteases that are complex-bound to serglycin. Together, these findings implicate serglycin in promoting apoptotic *versus* necrotic cell death.

Serglycin is a proteoglycan, *i.e.* it is composed of a "core protein" to which highly sulfated and thereby negatively charged polysaccharides of glycosaminoglycan type are attached (1, 2). Serglycin is a major component of secretory granules in many hematopoietic cell types (3–7) but has additionally been detected as a secretory product of non-hematopoietic cells (8). The high negative charge of serglycin enables tight electrostatic interactions with basic compounds that co-exist with serglycin in secretory granules (1). In support of a productive interaction between serglycin and other granule compounds *in vivo*, the ablation of serglycin expression in mice caused drastically impaired ability of mast cells to store numerous compounds, including mast cell-specific proteases of chymase, tryptase and carboxypeptidase type (7, 9). In addition, serglycin is essential for the storage of elastase and α-defensin in neutrophils (4, 10), granzyme B in cytotoxic T lymphocytes (3) and various α-granule compounds in platelets (5). Serglycin can also promote the delivery of cytotoxic proteases from effector into target cells (11).

Apoptosis is a tightly controlled process mainly initiated in the cytosol, in which latent pro-apoptotic compounds are activated through proteolytic cleavages, accompanied by neutralization of various anti-apoptotic compounds by proteolytic degradation (12, 13). Ultimately, initiation of the apoptotic program results in major morphological and biochemical events, including extensive DNA condensation, DNA fragmentation, formation of apoptotic bodies, and, finally, engulfment by phagocytic cells (12, 13). In contrast, necrosis and necrosis-like cell death are less well regulated processes, characterized by cell membrane permeabilization and release into the extracellular space of various danger signals that can cause inflammation (14).

The serglycin-dependent proteases stored in mast cell granules, as well as corresponding granule proteases of other hematopoietic cell types, are all stored in an enzymatically fully active form. This may create a potentially hazardous situation for the cell since leakage of serglycin-dependent proteases from the granules into the cytosol may cause proteolytic attack on various cytosolic proteins, including compounds that regulate cell death. As a proof of concept for this scenario, it was shown previously that serglycin affects mast cell death in response to direct targeting of secretory granules by using a lysosomotropic agent (15). However, it is not known whether serglycin regulates cell death in response to regimens other than those involving direct lysosomal/granule targeting, and the mechanism(s) by which serglycin affects cell death has not been clarified.

Here we investigated the role of serglycin in cell death induced by a panel of different regimens and we also sought to identify the mechanisms by which serglycin may regulate cell death. We show that wild type (WT) and serglycin$^{-/-}$ mast cells are approximately equally sensitive to cell death induced by a variety of regimens. However, the mechanism of cell death was dramatically affected by the absence or presence of sergly-

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cin, with WT cells dying predominantly by apoptosis whereas serglycin$^{-/-}$ cells died almost exclusively by necrosis. A dissection of the underlying mechanism revealed that the necrotic phenotype of serglycin$^{-/-}$ cells was linked to defective degradation of poly(ADP-ribose) polymerase-1 (PARP-1).[3] These findings implicate serglycin as a regulator of apoptosis *versus* necrosis.

EXPERIMENTAL PROCEDURES

Reagents—Cycloheximide (CHX), *cis*-Diammineplatinum(II) dichloride (cisplatin), actinomycin D, digitonin, 3-aminobenzamide (3-AB), Z-DEVD-FMK, cell permeable caspase-9 inhibitor, pepstatin A, Pefabloc SC, E64d were from Sigma-Aldrich. Calpeptin was from Merck (Darmstadt, Germany). Rabbit anti-ICAD (N-terminal) polyclonal antibody was from Millipore (Billerica, MA). Rabbit polyclonal to β-actin (I-19) and mouse monoclonal to AIF (E-1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to PARP-1 and rabbit monoclonal to ROCK-1 from Abcam (Cambridge, UK). Hamster anti-mouse CD95 and hamster IgG2 λ1 isotype control were from BD Pharmingen (San Jose, CA).

Animals—Serglycin$^{-/-}$ (7), mMCP-4$^{-/-}$ (16), mMCP-6$^{-/-}$ (17) as well as wild type (WT) control mice (8–18 weeks old) were all on C57BL/6J genetic background. All animal experiments were approved by the local ethical committee.

Bone Marrow-derived Mast Cells (BMMCs)—Bone marrow cells were prepared from femurs and tibiae of mice and were developed into BMMCs using medium containing IL-3 (18). Peritoneal-derived mast cells (PCMCs) were cultured as previously described (19).

Induction/Inhibition of Cell Death—Triplicates of 1 ml BMMCs (0.5 × 10^6 cells/ml) were transferred into individual wells of 24-well flat-bottomed plates, and were either left untreated or treated with either CHX (5 μg/ml), cisplatin (20 μg/ml), H$_2$O$_2$ (0.75 mM), 1 μg/ml of hamster anti-mouse CD95 (Fas) antibody, or hamster IgG2 λ1 isotype control (together with 10 ng/ml of actinomycin D) in complete culture medium, followed by incubation for different time periods. To assess the effect of various inhibitors on cell death, cells were preincubated with either of the following compounds: broad spectrum cysteine cathepsin inhibitor E64d (20 μM), the caspase-3/7 inhibitor Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK)(20 μM), cell permeable caspase-9 inhibitor (20 μM), calpeptin (1 μM), Pefabloc SC (0.1 mM), Pepstatin A (50 μM)), 3-methyladenine (500 μM), or 3-aminobenzamide (5 mM).

Measurement of Cell Viability—Cell viability was measured using the CellTiter-Blue® cell viability assay (Promega-Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. Fluorescence was recorded (Ex. 560 nm/Em. 590 nm) using a fluorescence microplate reader. To access cell viability by fluorescence microscopy we used a live/dead® viability/cytotoxicity kit (Molecular Probes-Invitrogen), following the instructions from the manufacturer.

NAD$^+$ and ATP Measurements—Intracellular NAD$^+$ and ATP levels were measured using kits from Abcam (Cambridge, UK) and PerkingElmer Life & Analytical Science B.V. (Groningen, The Netherlands), respectively.

Morphology of Cells—Transmission electron microscopy (TEM) was performed as previously described (20).

Flow Cytometry—Flow cytometry was performed using a FITC Annexin V apoptosis detection kit (BD Pharmingen) according to the protocol provided by the manufacturer. After staining, cells were analyzed using a FACScan® flow cytometer and the CELLQuest^{TM} 3.3 software (Becton Dickinson, San Jose, CA). Data from 10,000 events/sample were collected.

Western Blot Analysis—0.5–2 × 10^6 BMMCs were solubilized in SDS-PAGE sample buffer containing 5% dithiothreitol. Samples corresponding to equal numbers of cells were subjected to Western blot analysis as previously described (20). Membranes were scanned using an Odyssey Infrared Imager (Li-cor, Lincoln, NE).

Cytosolic Extract Preparation—BMMCs (10^6 cells) were collected by centrifugation (1,200 rpm, 8 min, 4 °C) in 1.5-ml Eppendorf tubes and then resuspended in 300 μl of ice-cold digitonin extraction buffer (10 μg/ml digitonin, 250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA). After 10 min of incubation on ice, cells were centrifuged (13,000 rpm; 2 min, 4 °C), and the supernatant was quickly removed.

DNA Fragmentation—BMMCs (10^6 cells) previously washed in PBS were incubated with 0.5 ml of lysing buffer (PBS, 0.2% Nonidet P-40, 0.9 mM AEBSF, 0.4 mM EDTA, 80 μM Bestatin, 12 μM pepstatin A, 12 μM E64d) for 10 min at 4 °C. Next, the supernatant was recovered after centrifugation (12,000 rpm, 20 min, 4 °C) and RNA was digested using RNase (0.1 mg/ml at 37 °C for 1 h) followed by proteinase K digestion (20 μg/ml at 37 °C for 1 h). DNA was precipitated by adding an equal volume of isopropanol, followed by incubation overnight at −20 °C, and centrifugation at 12,000 × g for 15 min (4 °C). The pellets were air-dried, resuspended in 20 μl Tris acetate EDTA buffer supplemented with 2 μl of sample buffer (0.25% bromphenol blue, 30% glycerol), and electrophoretically separated on 2% agarose gels containing GelRed^{TM} nucleic acid gel stain (Biotium, Hayward, CA) and visualized under ultraviolet transillumination.

Statistical Analyses—Statistical significance was tested using one-way analysis of variance (ANOVA) using Origin 7.5 software (OriginLab Corp., Northampton, MA). All data shown are from individual experiments, representative of several (up to 5) independent experiments. Statistical significances are indicated as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, and **** $p \leq 0.0001$.

RESULTS

Mast Cells Are Non-sensitive to TNF but Are Highly Sensitive to Cycloheximide-induced Cell Death—We first assessed whether mast cells (bone marrow-derived mast cells (BMMCs)) undergo cell death in response to TNF. To block TNF-induced activation of survival mechanisms, TNF (10–100 ng/ml) was added in combination with cycloheximide (CHX) at concentrations up to 50 μg/ml, *i.e.* CHX concentrations that

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[3] The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase-1; BMMC, bone marrow-derived mast cell; mMCP, mouse mast cell protease; CHX, cycloheximide; AnnV, Annexin V; EthD-1, ethidium homodimer-1.
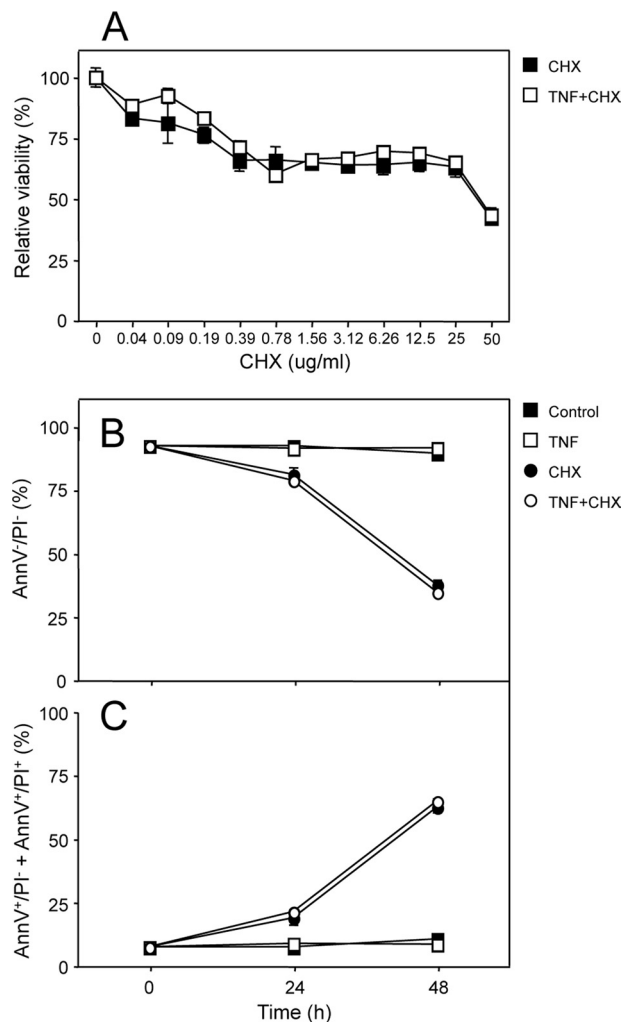
FIGURE 1. **Mast cells are non-sensitive to TNF but are highly sensitive to CHX-induced cell death.** *A*, WT bone marrow-derived mast cells (BMMCs) (0.5×10^6 cells/ml) were left untreated or treated with TNF (10 ng/ml) and different concentrations of CHX for 24 h. Cell viability was measured using a resazurin-based cell viability assay. *B* and *C*, WT BMMCs (0.5×10^6 cells/ml) were left untreated or treated with 10 ng/ml TNF and/or 5 μg/ml CHX. At the indicated time points, cells were collected, and cell death was measured by AnnV and PI staining using flow cytometry. Data are averages of triplicates $\pm$ S.E. (*B*) % of viable cells (AnnV$^-$/PI$^-$); (*C*) % of dead cells, apoptotic (AnnV$^+$/PI$^-$) plus necrotic (AnnV$^+$/PI$^+$) cells.

are non-toxic to most cell types. The combined TNF/CHX treatment was cytotoxic for mast cells. However, there was no difference in cytotoxicity when comparing cells treated with CHX only with the combined TNF/CHX treatment (Fig. 1). These results suggest that mast cells are resistant to TNF but are highly sensitive to cell death induced by CHX.

Serglycin Promotes Apoptosis Over Necrosis in Mast Cells—Next, we examined the role of serglycin by comparing the susceptibility of wild type (WT) and serglycin$^{-/-}$ cells to CHX. As seen in Fig. 2*A*, CHX caused cell death of both WT and serglycin$^{-/-}$ mast cells with comparable efficiency. However, when comparing the fraction of apoptotic (AnnV$^+$/PI$^-$) with the fraction of necrotic/late-stage apoptotic (AnnV$^+$/PI$^+$) cells, a striking difference between WT and serglycin$^{-/-}$ cells was noted: WT cells were almost exclusively AnnV$^+$/PI$^-$ (indicating apoptosis), while most of the non-viable serglycin$^{-/-}$ cells were double AnnV$^+$/PI$^+$ (indicating necrosis or late-stage

apoptosis)(Fig. 2, *A* and *B*). Importantly, non-viable serglycin$^{-/-}$ cells were AnnV$^+$/PI$^+$ at all time points investigated, *i.e.* they were not initially single AnnV-positive followed by subsequent positivity for PI. This suggests that the non-viable serglycin$^{-/-}$ cells were necrotic rather than late-stage apoptotic. These findings indicate that serglycin has a key role in promoting apoptotic cell death in mast cells and that the absence of serglycin results in predominant necrosis. In agreement with this, DNA fragmentation was clearly visible in WT cells, but was diminished in cells lacking serglycin (Fig. 2*C*).

Staining of the cells with calcein AM/ethidium homodimer-1 (EthD-1) revealed, as expected, strong calcein AM staining (sign of viability) in non-treated cells, but a gradual reduction in staining after induction of cell death (Fig. 3*A*). In agreement with the approximately equal sensitivity of WT and serglycin$^{-/-}$ cells to CHX treatment, comparable reduction of calcein AM staining was seen in WT and serglycin$^{-/-}$ cells. In contrast, EthD-1 staining was remarkably different. In WT cells, EthD-1 staining was, as expected (EthD-1 does not penetrate intact cell membranes), absent in non-treated cells. In treated WT cells, EthD-1 staining was relatively weak and diffusely distributed in large parts of the cells (Fig. 3*A*), suggesting staining of partially degraded DNA located outside of the cell nucleus in cells with a damaged cell membrane. In contrast, the nuclei of serglycin$^{-/-}$ cells stained strongly with EthD-1 even after prolonged treatment, indicating preserved chromosomal DNA and lost integrity of the cell membrane. The latter is in good agreement with the necrotic cell death and limited DNA fragmentation seen in non-viable serglycin$^{-/-}$ cells (see Fig. 2*C*).

To provide further morphological insight into the mechanism of cell death in WT *versus* serglycin$^{-/-}$ mast cells, ultrastructural analysis was performed. In agreement with previous reports (20), granules were present at approximately equal numbers in WT and serglycin$^{-/-}$ cells, but the granule ultrastructure was profoundly influenced by the absence of serglycin. As seen in Fig. 3*B*, WT granules contained dense core regions interspersed with electron-translucent areas, while granules from serglycin$^{-/-}$ cells showed an amorphous appearance with moderately electron-dense material distributed evenly within the entire granules. When treated with cell death-inducing agent, WT cells showed typical signs of apoptosis, including DNA condensation, and it was also noted that the cell membrane appeared intact (Fig. 3*B*). In contrast, treated serglycin$^{-/-}$ cells showed a typical necrotic appearance, as judged by loss of integrity of cell membranes and absence of DNA condensation. It is also notable that cell death of both WT and serglycin$^{-/-}$ cells was accompanied by a reduction in electron density of the storage granules, compatible with permeabilization of granule membranes and leakage of granule compounds (such as serglycin) into the cytoplasmic compartment.

Minor Role of Caspases and Calpains in CHX-induced Cell Death—To address the mechanism of mast cell death in response to CHX and to approach the mechanism by which serglycin affects cell death, we first investigated the role of caspases. Caspase-3/7 inhibition by Z-DEVD-FMK resulted in a marginal, but statistically significant prevention of cell death in response to CHX (Fig. 4), while a caspase-9 inhibitor had no
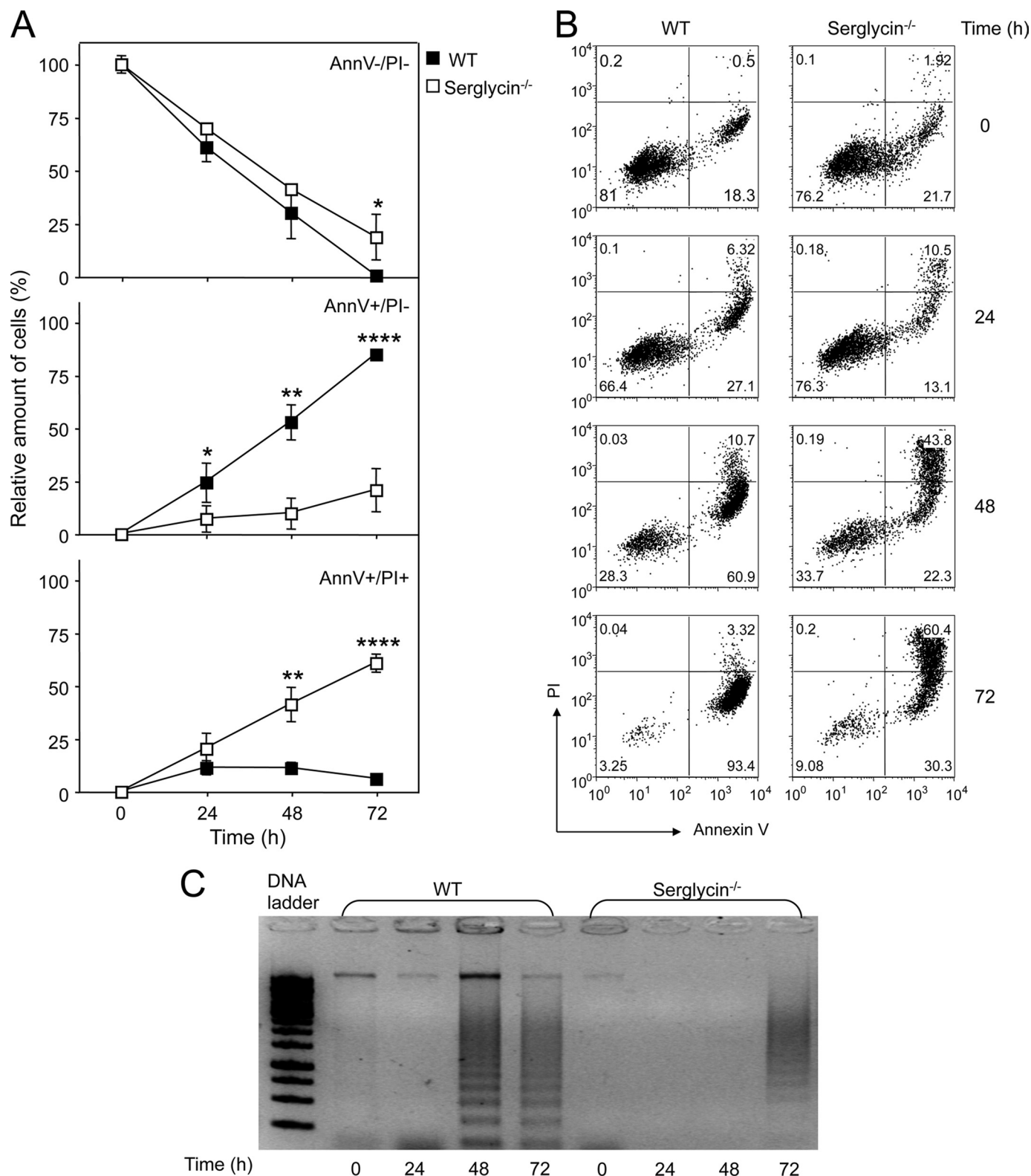
FIGURE 2. **Serglycin promotes apoptosis over necrosis in mast cells.** *A*, WT and serglycin$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were left untreated or treated with CHX (5 μg/ml). At the indicated time points, cells were collected, and cell death was measured by AnnV/PI staining using flow cytometry. Data are averages of triplicates $\pm$ S.E. *B*, dot plots from representative samples showing staining with AnnV (FL-1) and PI (FL-3). *C*, DNA degradation in response CHX. DNA was extracted from WT and serglycin$^{-/-}$ BMMCs at the indicated time points after addition of CHX (5 μg/ml), followed by agarose gel electrophoresis.

effect (Fig. 4). Hence, mast cell death in response to CHX is largely caspase-independent. Notably, the marginal prevention of cell death by Z-DEVD-FMK was only evident in WT cells,

with no effect of Z-DEVD-FMK on cells lacking serglycin. Previous findings have suggested a role of calpains in caspase-independent cell death (21), and we therefore investigated the
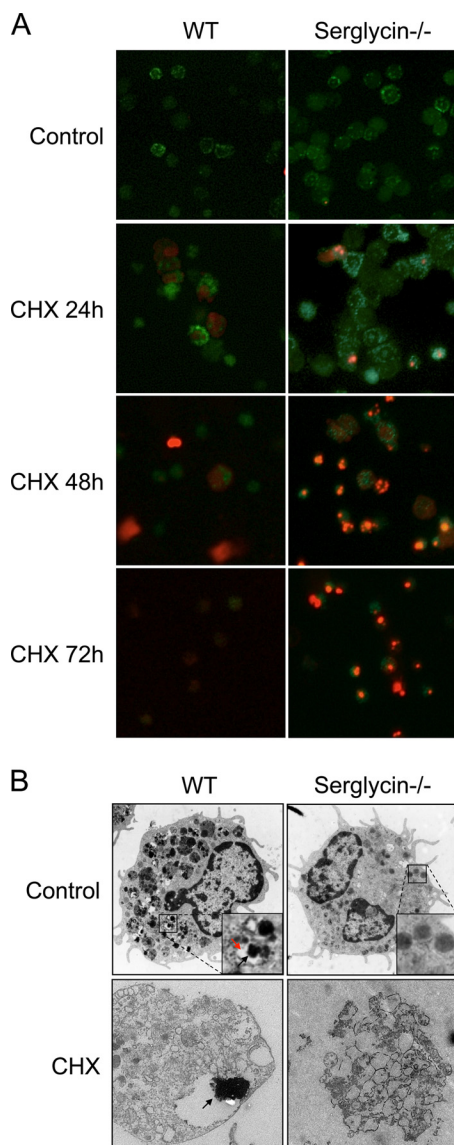
FIGURE 3. **Morphological effects of CHX-induced cell death in BMMCs.** *A,* WT and serglycin$^{-/-}$ BMMCs (0.5 × 10^6 cells/ml) were left untreated or treated with CHX (5 μg/ml). At the time points indicated, cytospin slides were prepared and stained with calcein-AM (live cells probe, *green*) and ethidium homodimer-1 (EthD-1) (dead cells probe, *red*). Analyses were performed by fluorescence microscopy (original magnification ×20). *B,* transmission electron microscopy analysis of untreated WT and serglycin$^{-/-}$ BMMCs (*upper panels*), showing dense core formation (*black arrow* in inset, WT), interspersed with electron translucent areas (*red arrow* in inset; WT) in WT cells, whereas the granule content of serglycin$^{-/-}$ cells has an amorphous appearance without dense core formation. The *lower panels* show typical morphology of non-viable WT and serglycin$^{-/-}$ cells. Note the extensive DNA condensation in WT cells (*arrow*). Note also the extensive signs of necrosis, *i.e.* decomposed cell membrane, in serglycin$^{-/-}$ cells. Original magnifications, ×12,500.

impact of this class of proteases on mast cell death. Calpain inhibition preserved cell viability to a minor but significant extent in WT cells but had, in contrast, no effect on the viability of serglycin$^{-/-}$ cells (Fig. 4). Cell death was not prevented by E64d, a cell membrane-permeable inhibitor of cysteine cathepsins, and there was no effect of pepstatin A, the latter an inhibitor of aspartic acid proteases (Fig. 4). Thus, although serglycin promotes caspase- and calpain-mediated apoptosis, caspase- and calpain-mediated pathways play only a marginal role in mast cell death in response to CHX.
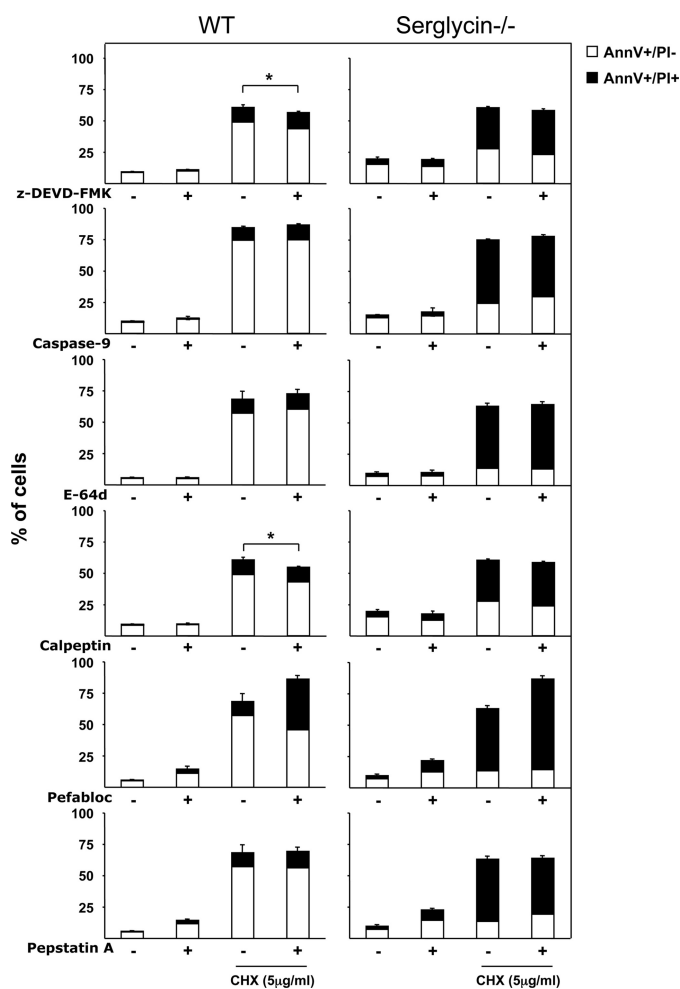
FIGURE 4. **Effect of protease inhibitors on cell death of WT and serglycin$^{-/-}$ mast cells.** WT and serglycin$^{-/-}$ bone marrow-derived mast cells (BMMCs) (0.5 × 10^6 cells/ml) were left untreated or treated for 30 min with 20 μM Z-DEVD-FMK, 20 μM cell permeable caspase-9 inhibitor, 20 μM E-64d, 1 μM calpeptin, 0.1 mM Pefabloc SC, or 50 μM pepstatin A, and were thereafter incubated for 48 h ± 5 μg/ml CHX as indicated. Cells were stained with AnnV and PI and analyzed by flow cytometry. Data are averages of triplicates ± S.E. The *open parts* of the bars represent apoptotic (AnnV$^+$/PI$^-$) cells, and the *filled parts* of the bars correspond to necrotic (AnnV$^+$/PI$^+$) cells.

Serglycin Promotes Apoptosis over Necrosis in Response to Cisplatin, Oxidative Stress, and Fas Ligation—Next, we assessed whether the ability of serglycin to promote apoptosis over necrosis is a general property of serglycin, independently on how cell death is induced. We therefore investigated the role of serglycin in cell death induced by different regimens. Cisplatin is a widely used chemotherapeutic drug that induces cell death in target cells, although the precise mechanism of cell death is not fully understood (22). Addition of cisplatin to mast cells caused cell death, with WT and serglycin$^{-/-}$ cells showing equal susceptibility (Fig. 5*A*). However, similar to treatment with CHX, WT cells underwent predominantly apoptotic cell death, whereas serglycin$^{-/-}$ cells were to a major extent necrotic. Also in response to oxidative stress, WT and serglycin$^{-/-}$ cells were equally susceptible (Fig. 5*A*). However, similar to treatment with CHX and cisplatin, WT cells were to a large extent apoptotic, whereas serglycin$^{-/-}$ cells underwent cell death with signs of necrosis (Fig. 5*A*).
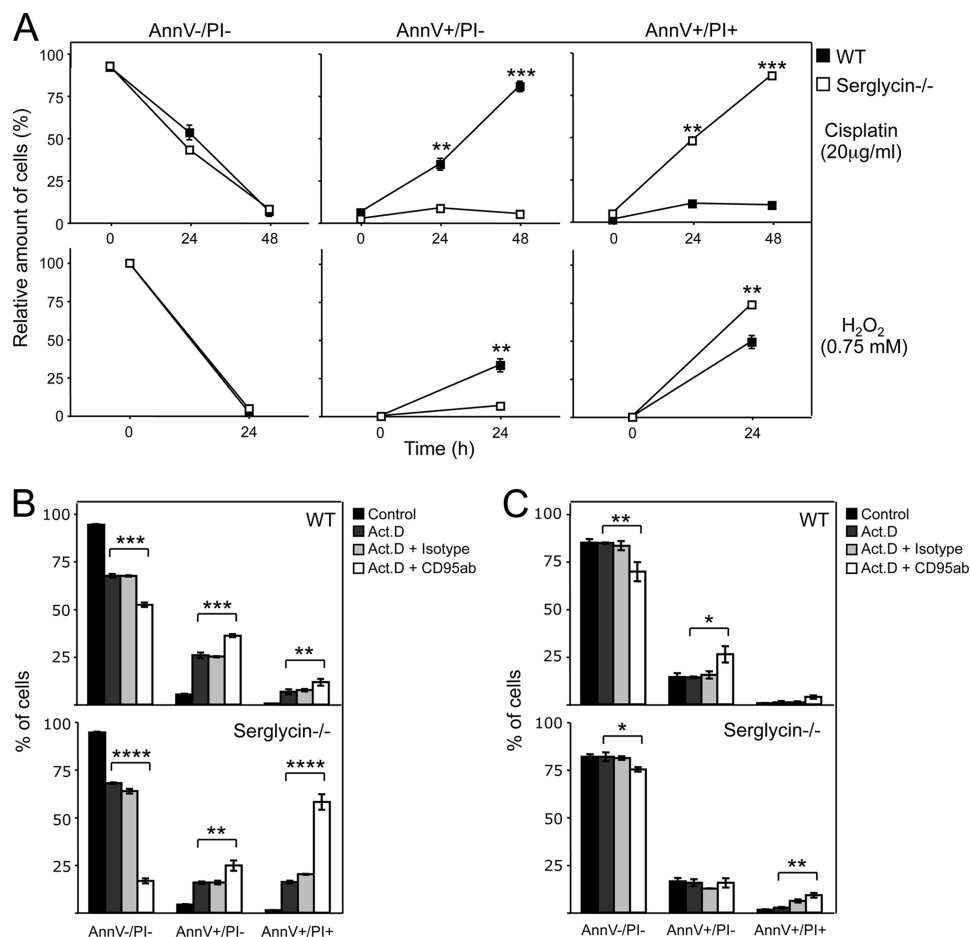
FIGURE 5. **Serglycin promotes apoptosis over necrosis in response to cisplatin, oxidative stress, and Fas ligation.** *A*, WT and serglycin^{−/−} bone marrow-derived mast cells (BMMCs) (0.5 × 10^6 cells/ml) were treated with 20 μg/ml cisplatin or 0.75 mM H₂O₂. At the time points indicated, cells were stained with Annexin V (AnnV)/PI and analyzed by flow cytometry. Data are averages of triplicates ± S.E. *B* and *C*, WT and serglycin^{−/−} BMMCs maintained for 11 months (*B*) or peritoneal cell-derived mast cells (PCMCs) (*C*) (0.5 × 10^6 cells/ml) were treated with 1 μg/ml of agonistic CD95 antibody or isotype control ± 5 ng/ml actinomycin D. At the time points indicated, cells were stained with AnnV/PI. Data are averages of triplicates ± S.E.

Mast cells have previously been shown to express Fas (CD95) on their surface and to undergo cell death in response to Fas ligation (23). To investigate if serglycin influences the cell death pathway in response to Fas ligation, we added an agonistic anti-CD95 antibody to WT and serglycin^{−/−} BMMCs and assessed the extent and type of cell death. To block Fas-induced survival pathways, actinomycin D was added together with the agonistic anti-CD95 antibody (23). Fas ligation caused only minimal cell death of 4-week-old BMMCs, compatible with low expression of Fas in cells at this stage of mast cell maturation (24). In contrast, BMMCs cultured for 11 months showed significant cell death in response to Fas ligation (Fig. 5*B*), in agreement with high expression of Fas in more mature mast cells (24). Strikingly, whereas Fas ligation-susceptible WT cells were predominantly apoptotic, susceptible serglycin^{−/−} cells were predominantly necrotic (Fig. 5*B*). To pursue these findings by investigating the role of serglycin in Fas-mediated cell death of terminally differentiated mast cells, we developed peritoneal cell-derived mast cells (PCMCs)(19) from WT and serglycin^{−/−} mice and investigated if they were sensitive to Fas ligation. Indeed, WT PCMCs underwent cell death in response to Fas ligation with characteristics of apoptosis (Fig. 5*C*), in agreement with previous studies showing that peritoneal mast cells express Fas (23). In contrast, there was no sign of apoptotic cell death in anti-CD95-treated serglycin^{−/−} cells. Rather, the small fraction of serglycin^{−/−} PCMCs that underwent cell death was necrotic (Fig. 5*C*).

PARP-1 Is Preserved during Death of serglycin^{−/−} Cells—To further elucidate the mechanism by which serglycin regulates cell death, we assessed whether serglycin-deficiency was associated with differential processing of proteins participating in the apoptotic process. First, we analyzed for processing of PARP-1, a protein involved in DNA repair (25). In response to apoptotic stimuli, PARP-1 is degraded, resulting in defective DNA repair and, consequently, DNA fragmentation. In situations where PARP-1 degradation is defective, non-restrained PARP-1 activity may cause energy depletion and this, in turn, can lead to necrosis (25). Thus, defective PARP-1 degradation may be a potential explanation for the necrotic cell death in serglycin^{−/−} cells and we therefore decided to examine if the absence of serglycin could influence the processing/levels of PARP-1 in response to cell death. As seen in Fig. 6*A*, PARP-1 was efficiently degraded in WT cells, but was remarkably preserved in serglycin^{−/−} cells after induction of cell death. It was also evident that the baseline levels of PARP-1 (prior to treatment) were higher in serglycin^{−/−} BMMCs than in WT counterparts (Fig. 6*A*).
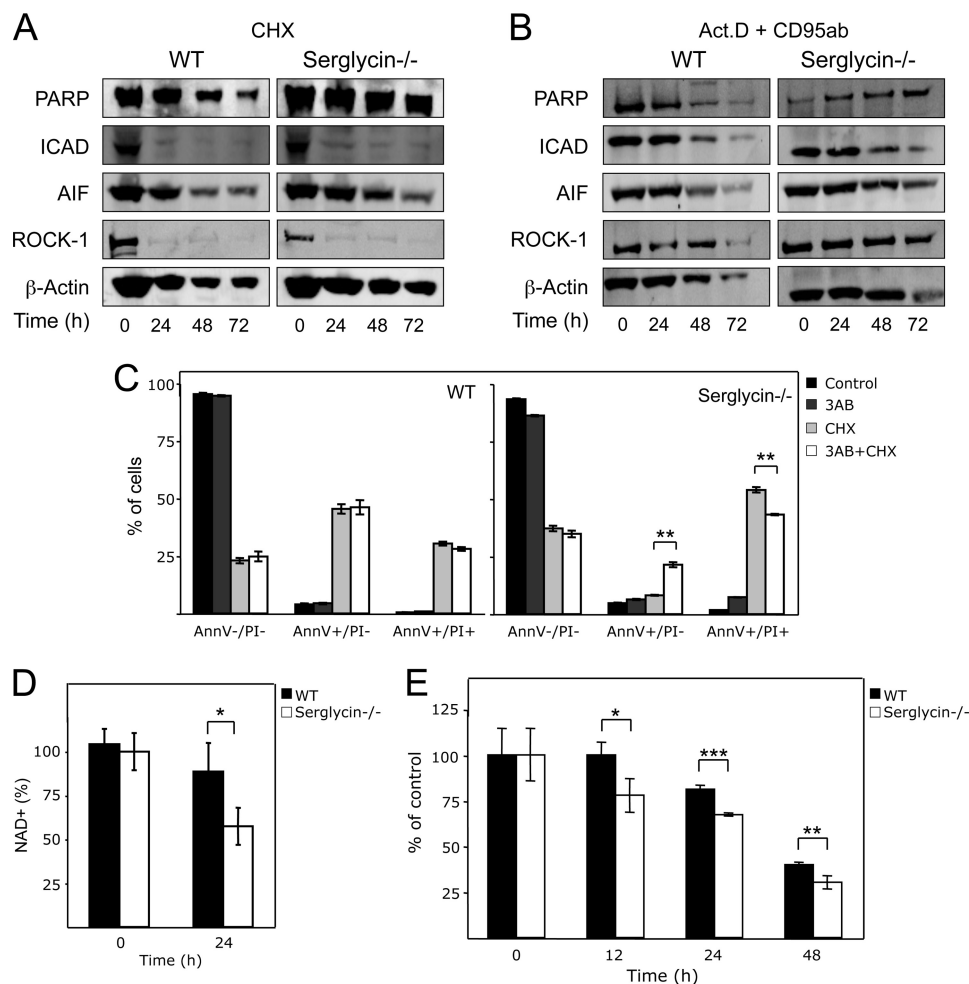
FIGURE 6. **PARP-1 is preserved in serglycin$^{-/-}$ cells.** WT and serglycin$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were left untreated or treated with either CHX (5 µg/ml) (*A*), or 1 µg/ml of CD95 antibody in combination with 5 ng/ml actinomycin D (*B*). At the time points indicated, samples corresponding to equal numbers of cells were subjected to Western blot analysis for PARP-1, inhibitor of caspase-activated DNase (*ICAD*), apoptosis-inducing factor (AIF), Rho-associated coiled-coil protein kinase 1 (*ROCK-1*), with β-actin as loading control. *C*, PARP-1 inhibition reverses necrosis in serglycin$^{-/-}$ BMMCs. WT and serglycin$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were either left untreated or treated with 5 mM 3-Aminobenzamide (3-AB), followed by incubation with 5 µg/ml CHX. After 48 h of incubation, cells were stained with Annexin V (AnnV)/PI. *D*, NAD$^+$ levels in untreated and CHX-treated (24 h) WT and serglycin$^{-/-}$ BMMCs. Data are averages of triplicates treatments ± S.E. *E*, ATP levels in untreated and CHX-treated WT and serglycin$^{-/-}$ BMMCs. Data are averages ± S.E. (*n* = 4).

We also investigated whether the differential mode of cell death in WT *versus* serglycin$^{-/-}$ cells was associated with differential processing of the inhibitor of caspase-activated DNase (ICAD). However, ICAD processing was similar in WT and serglycin$^{-/-}$ cells (Fig. 6*A*). Apoptosis-inducing factor (AIF) is known to be a major player in caspase-independent cell death (26). Since our data indicate that BMMC apoptosis in response to CHX is largely caspase-independent (see Fig. 4), we investigated if AIF processing occurs during CHX-induced mast cell death and if AIF processing is different in WT *versus* serglycin$^{-/-}$ cells. As shown in Fig. 6*A*, AIF was degraded to a similar extent in WT and serglycin$^{-/-}$ cells, and truncated AIF was not detectable following induction of apoptosis. We also analyzed for differential processing of Rho-associated coiled-coil protein kinase 1 (ROCK-1), a protein that has a prominent role in the degradation of the cytoskeleton during apoptosis. However, ROCK-1 was degraded to a similar extent in WT and serglycin$^{-/-}$ cells (Fig. 6*A*).

Next, we tested whether PARP-1 preservation also could explain the differential mode of cell death of WT and sergly-

cin$^{-/-}$ mast cells in response to Fas ligation. As conventionally cultured BMMCs (3–10 weeks) were minimally sensitive to Fas ligation (see above), we used long-term BMMC cultures (11 months) for this purpose. In agreement with the findings in CHX-treated cells, PARP-1 was efficiently degraded in WT cells in response to Fas ligation, but was remarkably preserved in cells lacking serglycin (Fig. 6*B*). Together, these data indicate that serglycin has a prominent role in the regulation of PARP-1 levels in response to cell death induction.

PARP-1 Inhibition Reverses Necrosis in Serglycin-deficient Cells—To further evaluate the possibility that the necrotic cell death of serglycin-deficient cells is due to defective PARP-1 degradation, we treated WT and serglycin$^{-/-}$ cells with the PARP-1 inhibitor 3-aminobenzamide (*3-AB*). Treatment of serglycin$^{-/-}$ mast cells with PARP-1 inhibitor resulted in a significant increase in the proportion of apoptotic cells, along with a reduced population of necrotic cells (Fig. 6*C*). In contrast, PARP-1 inhibition had no effect on the ratio of apoptotic/necrotic WT cells. Together, these findings indicate that the necrotic phenotype of cells lacking serglycin can be explained
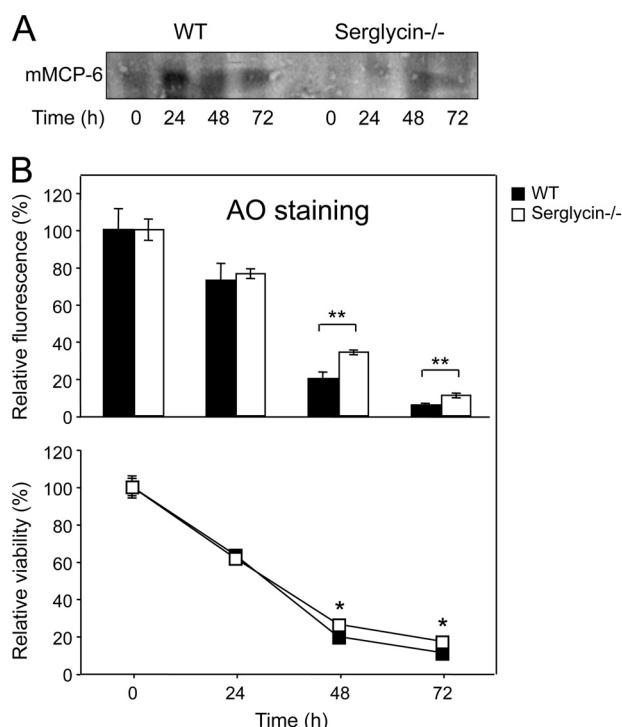
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FIGURE 7. **Secretory granule permeabilization accompanies mast cell death.** WT and serglycin$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were left untreated or treated with CHX (5 μg/ml). *A*, at the indicated time points, cells were collected and cytosolic extracts were prepared and subjected to Western blot analysis for mouse mast cell protease 6 (mMCP-6). *B*, staining of acidic cellular compartments was performed using acridine orange (AO; *upper panel*). The corresponding viability of cells is shown in the *lower panel*. Data are averages of triplicates ± S.E.

by preservation of PARP-1. In agreement with PARP-1-mediated energy depletion being a cause of necrotic cell death in cells lacking serglycin, NAD$^+$ (Fig. 6D) and ATP (Fig. 6E) were depleted at a higher rate in serglycin$^{-/-}$ than in WT cells after induction of cell death.

Secretory Granule Permeabilization Accompanies Cell Death in Mast Cells—Since serglycin is a secretory granule compound whereas the pro-apoptotic machinery is largely cytosolic, a possible scenario behind the apoptosis-promoting effect of serglycin is that it, during the cell death process, escapes from the granules into the cytosol. Indeed, the reduction in electron density of granules after cell death induction (see Fig. 3B) supports this scenario. To investigate this possibility further, we first tested whether mast cell death was associated with a reduction in acridine orange (AO) staining. AO is a cell-permeable dye that, when present in acidic compartments such as lysosomes and secretory granules, will give intense fluorescence at 650 nm. When the acidic compartments are damaged, the resulting rise in pH will cause reduced AO fluorescence. Hence, a reduction in AO fluorescence can be used as a means of monitoring lysosome/secretory granule integrity. As shown in Fig. 7B, mast cell death was accompanied by reduced AO staining, suggesting secretory granule permeabilization. Granule permeabilization occurred in both WT and serglycin$^{-/-}$ cells, but was somewhat more pronounced in WT cells. To provide further evidence for leakage of granule compounds into the cytosol, we analyzed cytosolic extracts for the presence of mouse mast cell protease 6 (mMCP-6), a mast cell-specific granule protease (27). Indeed,

mMCP-6 protein was detectable in the cytosol of CHX-treated- but was low in non-treated cells (Fig. 7A). As expected, mMCP-6 protein was undetectable in cytosolic extracts from serglycin$^{-/-}$ cells, either in treated or non-treated cells (Fig. 7B), in agreement with a dependence of mMCP-6 on serglycin for storage in granules (7, 20). These findings indicate that mast cell death is accompanied by permeabilization of the secretory granules and leakage of granule compounds into the cytosol.

A Role for Serglycin-dependent Tryptase mMCP-6 in Regulation of Mast Cell Apoptosis—As noted above (see Introduction), a major physiological role of serglycin is to promote the storage of various serine proteases within the mast cell secretory granules. To further outline the mechanism by which serglycin promotes apoptosis we asked whether the apoptosis-promoting effect of serglycin may be related to downstream effects mediated by the serine proteases that are stored in complex with serglycin. First, we examined whether a general serine protease inhibitor (Pefabloc SC) could affect the mode of cell death, *i.e.* apoptosis or necrosis. As shown in Fig. 4, global serine protease inhibition favored necrotic over apoptotic cell death in WT cells, clearly in line with a role for serglycin-bound serine proteases in promoting apoptosis over necrosis. Major serglycin-dependent serine proteases include the chymase mMCP-4 and the tryptase mMCP-6 (7). To examine the role of these proteases individually we compared the susceptibility and mechanism of cell death in WT, mMCP-4$^{-/-}$ and mMCP-6$^{-/-}$ mast cells. As shown in Fig. 8A, WT, mMCP-4$^{-/-}$ and mMCP-6$^{-/-}$ cells were approximately equally sensitive to cell death. Similar to WT cells, mMCP-4$^{-/-}$ mast cells showed clear signs of apoptosis whereas, in contrast, mMCP-6$^{-/-}$ cells mimicked serglycin-deficient cells by predominantly undergoing necrotic cell death (Fig. 8, A and B). As in serglycin$^{-/-}$ cells, defective apoptosis in mMCP-6$^{-/-}$ cells was supported by a markedly reduced extent of DNA fragmentation in comparison with the pattern seen in WT and mMCP-4$^{-/-}$ mast cells (Fig. 8C). It is also clear that, similar to cells lacking serglycin, baseline PARP-1 levels were higher in mMCP-6$^{-/-}$ as compared with WT cells (Fig. 8D). Further, although preservation of intact PARP-1 after induction of cell death was not as evident in mMCP-6$^{-/-}$ as in serglycin$^{-/-}$ cells, we noted a pronounced preservation of a ~25 kDa PARP-1 cleavage product, most likely corresponding to the 24-kDa DNA-binding PARP-1 fragment (28, 29). Preservation of this PARP-1 fragment was also seen in serglycin$^{-/-}$ cells, but was not apparent in WT or mMCP-4$^{-/-}$ cells. Together, these findings indicate that mMCP-6, at least partly, accounts for the effects of serglycin on apoptosis *versus* necrosis in mast cells.

DISCUSSION

In mast cells, but also in cytotoxic T lymphocytes and neutrophils, a major function of serglycin is to promote the storage of a number of neutral serine proteases in secretory granules. These proteases are stored in large quantities and are present in processed form, *i.e.* with their activation peptides cleaved off. Within the granule compartment, their enzymatic activities are kept low due to the prevailing acidic pH. However, if the granule membrane becomes damaged (permeabilized), serglycin and those proteases that are attached to it (as well as other,
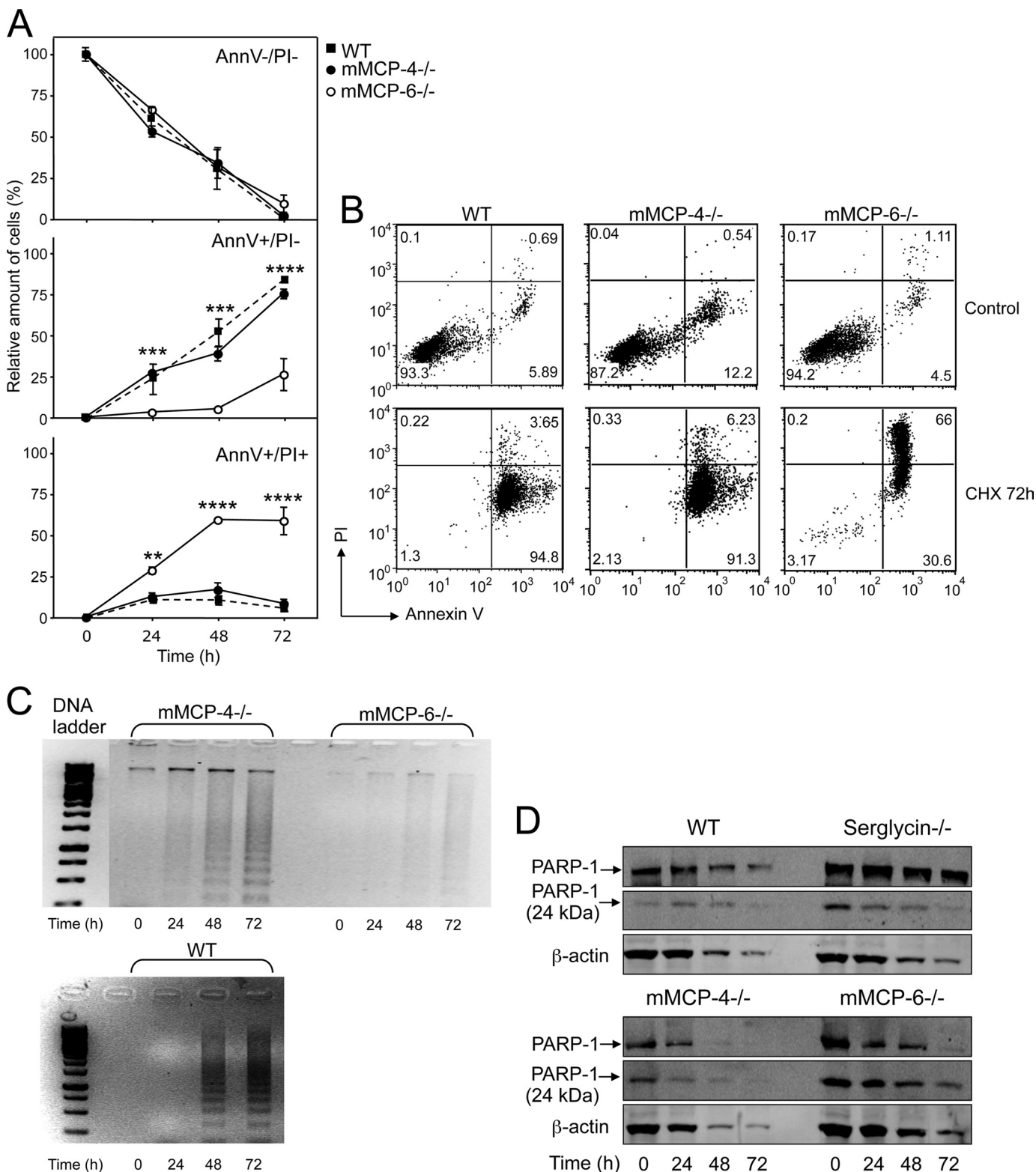
FIGURE 8. **A role for serglycin-dependent tryptase mouse mast cell protease (mMCP) 6 in regulation of BMMC apoptosis.** *A*, WT, mMCP-4$^{-/-}$ or mMCP-6$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were left untreated or treated with CHX ($5~\mu g$/ml). At the indicated time points, cells were collected and stained with AnnV/PI. Data are averages of triplicates $\pm$ S.E. *B*, dot plots from representative samples showing staining with AnnV (FL-1) and PI (FL-3). *C*, DNA degradation during cell death. DNA was extracted from WT, mMCP-4$^{-/-}$, and mMCP-6$^{-/-}$ BMMCs at the indicated time points after addition of CHX ($5~\mu g$/ml), followed by agarose gel electrophoresis. *D*, WT, serglycin$^{-/-}$, mMCP-4$^{-/-}$ and mMCP-6$^{-/-}$ BMMCs (0.5×10^6 cells/ml) were left untreated or treated with CHX ($5~\mu g$/ml). At the time points indicated, samples corresponding to equal numbers of cells were subjected to Western blot analysis for PARP-1, with β-actin as loading control.

non-serglycin dependent proteases) may escape from the granules into the cytosol and, in the neutral milieu of the cytosol, the granule proteases will become fully enzymatically active. Thus, serglycin-associated granule proteases entering the cytosol will have the potential to cause substantial proteolytic events if unrestricted, *e.g.* to proteolytically activate components of the apoptosis program. In this context, it is important to note that cells have an elaborate system of cytosolic protease inhibitors to protect the cell from damage caused by proteases escaping from lysosomal/granule compartments (30). However, if the extent of protease release from lysosomal/granule compartments exceeds the neutralizing capacity of these cytosolic protease inhibitors, proteolytic damage may occur (reviewed in Ref. 31). Serglycin entering the cytosol may also have direct (independently on its bound proteases) impact on various cytosolic components, *e.g.* on components involved in apoptosis.

In strong support of the scenario outlined above, we here show that serglycin has a striking impact on cell death by strongly promoting apoptosis over necrosis. Importantly, the strong apoptosis-promoting effect of serglycin was apparent in response to a multitude of different cell death-inducing agents, including both pharmacological and physiologically relevant stimuli, suggesting that serglycin has a general role in promoting apoptotic over necrotic cell death. It is also important to stress that neither of the cell death-inducing agents used in this study are known to have direct lysosome/granule-permeabilizing properties, implying that the granule damage that is required in order for serglycin to enter the cytosol is a natural event occurring secondary to induction of the apoptotic program. The latter notion is also supported by a number of previous studies showing that lysosome damage accompanies cell death induced in a variety of cell types, and in response to a panel of different cell death-inducing regimens (32–36).

The exact mechanism by which serglycin promotes apoptosis over necrosis is intriguing. Here we show that while PARP-1 was efficiently degraded in WT cells in response to cell death induction, PARP-1 levels were preserved in cells lacking serglycin. Most likely, the preserved PARP-1 is explained by defective PARP-1 degradation and, consequently, PARP-1 is likely to be degraded by a serglycin-dependent mechanism. When PARP-1 degradation in response to cytotoxic agents is defective, sustained PARP-1-mediated DNA repair will deplete cellular sources of energy leading to necrosis (37). Thus, it appears likely that the necrotic cell death seen in serglycin$^{-/-}$ cells is associated with unrestricted PARP-1-mediated DNA repair. In agreement with this scenario, DNA degradation was markedly defective in serglycin$^{-/-}$ cells. Moreover, inhibition of PARP-1 in serglycin$^{-/-}$ cells reversed the necrotic cell death, mirrored by a higher proportion of apoptotic cells. In contrast, PARP-1 inhibition did not alter the ratio of apoptotic/necrotic WT mast cells. Together, these findings argue that the necrotic cell death occurring in the absence of serglycin may be explained by defective PARP-1 degradation.

A crucial question is whether the apoptosis-promoting effect of serglycin is due to direct effects of serglycin on the apoptotic machinery, or mediated by downstream effects of those serine proteases that are stored in complex with serglycin. In mast cells, major serglycin-associated proteases include mMCP-4

and mMCP-6 (7), and we therefore assessed whether the phenotype seen in serglycin$^{-/-}$ cells was mimicked by cells lacking either of these. Indeed, we show that mMCP-6$^{-/-}$ mast cells, but not cells lacking mMCP-4, display similar defects in apoptosis as seen in serglycin$^{-/-}$ cells. Thus, it is likely that mMCP-6 accounts, at least partly, for the apoptosis-promoting effect of serglycin. mMCP-6, similar to other mammalian mast cell tryptases, has a unique tetrameric structure with all of its active sites facing a narrow central pore (38). Due to this macromolecular organization, the active sites of mMCP-6 are inaccessible to all endogenous protease inhibitors. Thus, if escaping from granules to the cytosol, mast cell tryptases such as mMCP-6 may exert proteolytic action on cytosolic proteins, unhindered by cytosolic protease inhibitors. Thereby, mMCP-6 is well suited for having a major role in executing degradation of cytosolic proteins, a notion that is clearly in line with the findings presented in this study.

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